

**PULP AND PAPER MILL EFFLUENTS AS A SOURCE
OF CYTOCHROME P4501A1 INDUCERS IN FISH OF
THE MIRAMICHI RIVER, NEW BRUNSWICK**

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ABSTRACT

Melanson, P.L., Chiasson, A.G. and Courtenay, S.C. 2004. Pulp and paper mill effluents as a source of cytochrome p4501A1 inducers in fish of the Miramichi River, New Brunswick. Can. Manuscrip. Rep. Fish. Aquat. Sci. 2709: xi + 42 p.

Previous studies have reported elevated concentrations of cytochrome P4501A1 (CYP1A1) mRNA in the livers of fish from the industrialized Miramichi River estuary suggesting exposure to certain organic contaminants. These studies implicated a bleached kraft pulp and paper mill (BKM) and a groundwood mill (GM) which discharge effluents into the upper estuary. In a series of three experiments, we tested the hypothesis that these effluents contain CYP1A1 inducers for fish in concentrations sufficient to explain the induction reported in wild fish.

Immature rainbow trout (*Oncorhynchus mykiss*) exposed for 133h to concentrations of secondary treated BKM effluent equal to or greater than 12% produced significant 2- to 10-fold CYP1A1 mRNA induction over controls. Primary treated GM effluent was lethal to trout at concentrations above 3% and no CYP1A1 mRNA induction was observed in the 3% exposed group.

Mature Atlantic tomcod (*Microgadus tomcod*) were exposed to effluent dilutions under static conditions for 6-8 d. Pure BKME produced significant 6-fold hepatic CYP1A1 mRNA induction (sexes combined) but no significant response was observed to 1% BKME or 0.2% GME. Similarly, in a second exposure significant 6-fold induction was elicited in males by 10% BKME and also by 3% GME but not by lower concentrations of either effluent. However, in a third exposure no significant induction of either CYP1A1 mRNA or EROD enzyme activity (another measure of CYP1A1 induction) was observed in females exposed to doses of 0.01 – 10% BKME or 0.03 – 3% GME. Plasma concentrations of vitamins E (tocopherol) and A (didehydroretinyl palmitate (DP) and retinyl palmitate (RP)) were also measured as indicators of oxidative stress in the second exposure and showed significant depression of DP in tomcod exposed to 0.3% and 3% GME, but no significant response to BKME.

Finally, Atlantic tomcod were caged for 10 d within the 1% effluent plumes of the two pulp and paper mills and at an unexposed reference site 5 km upstream. Significant CYP1A1 mRNA induction, relative to control fish held in the laboratory, was

observed at the BKM (5.3-fold) and at the GM (7.4-fold) but also at the unexposed reference site (4.4-fold). Blood plasma concentrations of DP were significantly lower in tomcod caged at the GM and upriver reference sites, but not BKM site, than in the laboratory control group. However, neither RP nor tocopherol concentrations showed similar depression.

We conclude that at the time of these experiments (1994-1995) effluents of the two Miramichi pulp mills contained CYP1A1 inducers for fish at least some of the time. However, the relatively small responses elicited by high effluent concentrations in laboratory exposures and the similar degrees of induction in fish caged within and beyond pulp mill effluent in the river suggest that the pulp mill effluents were not the sole or perhaps even the principal source of CYP1A1 inducers for fish in the Miramichi River.

RÉSUMÉ

Melanson, P.L., Chiasson, A.G. and Courtenay, S.C. 2004. Pulp and paper mill effluents as a source of cytochrome P4501A1 inducers in fish of the Miramichi River, New Brunswick. Can. Manuscrip. Rep. Fish. Aquat. Sci. 2709 : xi + 42 p.

Les études précédentes ont indiqué des concentrations élevées de cytochrome P4501A1 (CYP1A1) ARNm dans les foies des poissons de l'estuaire industrialisé de la rivière Miramichi suggérant l'exposition à certains contaminants organiques. Ces études ont impliqué une usine à pâte kraft blanchie (PKB) et une usine à copeaux qui déchargent des effluents dans la partie supérieure de l'estuaire. Dans une série de trois expériences, nous avons évalué l'hypothèse que ces effluents contiennent des inducteurs du système CYP1A1 aux concentrations suffisantes pour expliquer l'induction observée dans les poissons indigènes. Chez la truite arc-en-ciel juvénile (*Oncorhynchus mykiss*) exposée pendant 133h aux concentrations d'effluents provenant d'un traitement secondaire de PKB égal ou supérieur à 12%, nous avons observé une induction significative CYP1A1 ARNm de 2-10 fois au-dessus des témoins. L'effluent avec traitement primaire provenant de l'usine à copeaux était mortel à la truite aux concentrations au-dessus de 3% et aucune induction du système CYP1A1 ARNm n'a été observée dans le groupe exposé aux effluents à copeaux de 3%.

Les poulamons atlantique (*Microgadus tomcod*) ont été exposés aux dilutions d'effluents dans des conditions statiques pendant 6-8 jours. L'effluent à pâte kraft blanchie (EPKB) pure a produit une induction hépatique significative du système CYP1A1 ARNm de 6-fois celle des témoins (sexes combinés) mais aucune réponse significative a été observée à 1% EPKB ou à 0,2% d'effluent à copeaux. De même, dans la deuxième exposition des poissons à l'effluent, une induction significative de 6-fois a été obtenue chez les mâles exposés à 10% EPKB ainsi que 3% d'effluent à copeaux mais aucune réponse significative a été élicitée par des concentrations inférieures de l'un ou l'autre effluent. Cependant, dans une troisième exposition des poissons à l'effluent, nous n'avons observé aucune induction significative du système CYP1A1 ARNm ou d'activité enzymatique d'EROD (une autre mesure d'induction du système CYP1A1) chez les femelles exposées aux doses de 0,01 - 10% EPKB ou de

0,03 - 3% d'effluent à copeaux. Les concentrations plasmatiques de vitamine E (tocophérol) et de vitamine A (didéhydrorétinyl palmitate (DP) et rétinyle palmitate (RP)) ont été également mesurées comme indicateurs de stress oxydatif dans la deuxième exposition des poissons à l'effluent. Nous avons observé une diminution significative du taux de DP chez le poulamon exposé à 0,3% et à 3% d'effluent à copeaux mais aucune réponse à EPKB.

Finalement, les poulamons atlantique ont été mis en cage sur le terrain pendant 10 jours directement dans l'effluent de 1% rejeté par les deux usines de transformation de bois et à un site témoin non-exposé 5 kilomètres en amont. Nous avons observé une induction significative de CYP1A1 ARNm, relatif aux poissons témoins tenus en laboratoire, de 5,3 fois dans le cas d'EPKB et 7,4 fois dans le cas d'effluent à copeaux et de 4,4 fois chez les poissons encagés au site témoin, toutes en comparaison avec les poissons témoins tenus en laboratoire. Les concentrations plasmatiques du DP étaient sensiblement inférieures chez le poulamon encagé au site de l'usine à copeaux et au site témoin en amont mais pas à l'emplacement de l'usine à PKB, comparé au groupe témoin en laboratoire. Cependant, ni le RP ni les concentrations en tocophérol ont donné une diminution semblable.

Nous concluons qu'à l'heure actuelle des expériences (1994-1995) les effluents des deux usines de transformation de bois de la Miramichi contenaient des inducteurs de CYP1A1 capable de provoquer au moins une partie du temps une réponse physiologique chez les poissons. Cependant, les réponses relativement petites aux concentrations d'effluents élevées soient en laboratoire ou sur le terrain dans les rejets des usines indiquent que les effluents ne sont pas les seuls, ni même la source principale, des inducteurs CYP1A1 chez les poissons de la rivière Miramichi.

1.0 INTRODUCTION

A superfamily of enzymes known as cytochrome P450 is recognized for its function in the metabolism of endogenous and exogenous substrates including xenobiotics of environmental concern (Stegeman and Hahn 1994). Cytochrome P4501A (CYP1A) is a subfamily which contains three known genes (Nelson *et al.* 1996) which respond to some of the more prevalent and harmful anthropogenic organic contaminants. They include co-planar polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-furans (PCDFs) and dioxins (PCDDs). Two of the three CYP1A genes have been identified in fish, namely CYP1A3 and CYP1A1. Activation of these genes and production of CYP1A, referred to as induction, is a commonly used biomarker and early warning system of pollution exposure (Addison 1984; Payne *et al.* 1987; Goksoyr and Forlin 1992).

Several studies demonstrate significant CYP1A expression in environmentally exposed fish collected from polluted waterways (Payne and Penrose 1975; Stegeman *et al.* 1987; Van Veld *et al.* 1990). The vast majority of these studies quantify the CYP1A gene expression by measuring the catalytic activity of CYP1A-encoded enzymes such as ethoxyresorufin-O-deethylase (EROD) or by immunodetection of CYP1A proteins (Goksoyr 1985; Stegeman *et al.* 1987). These biomarkers may be modulated by a number of biological factors such as reproductive state, inhibition at high substrate concentrations or degradation by other contaminants (Courtenay *et al.* 1999). Measurement of induction at the transcriptional level (i.e., CYP1A mRNA) minimizes some of these potential interferences which occur at later stages in induced gene expression (Haasch *et al.* 1993).

The Atlantic tomcod (*Microgadus tomcod*) is a common estuarine teleost along the northeastern coast of North America with spawning populations extending from Labrador to the Hudson River, New York (Bigelow and Schroeder 1953). Characteristics that render the tomcod an excellent sentinel species include a bottom-dwelling existence and benthic diet, movements limited to the area of its natal estuary, abundance, and a large, extremely lipid-rich liver (Courtenay *et al.* 1995). Furthermore,

tomcod appear to show greater CYP1A1 mRNA inducibility than do several other species of Atlantic coast estuarine fishes (Wirgin *et al.* 1996).

CYP1A1 mRNA expression in tomcod has been used as a biomarker of exposure to organic xenobiotics in Atlantic estuaries of North America. Tomcod from the Miramichi River, New Brunswick, showed higher hepatic levels of CYP1A1 mRNA (Wirgin *et al.* 1994) and higher body burdens of organic contaminants (Courtenay *et al.* 1999) than tomcod from less industrialized estuaries. The largest anthropogenic impact on this estuary is two pulp and paper mills which discharge effluent into the upper estuary. Support for the hypothesis that these effluents were responsible for elevated CYP1A1 mRNA levels in tomcod came from an experiment in which tomcod caged near one of the two mills, a bleached kraft mill, showed significantly higher levels of CYP1A1 mRNA than was seen in fish caged either upstream or downstream of the mill (Courtenay *et al.* 1993). However, a number of other potential sources of CYP1A1 inducers are found in the same area of the Miramichi estuary including municipal wastewater discharges and a former wood treatment facility (Zitko *et al.* 2000). Therefore it remains to be demonstrated that the pulp mill effluents are responsible for CYP1A1 mRNA induction in Miramichi River tomcod.

In this study we tested the hypothesis that effluents from the bleached kraft mill (BKM) and the groundwood mill (GM) discharging into the Miramichi estuary are sources of CYP1A1 inducers for fish. Hepatic CYP1A1 mRNA concentrations were quantified in juvenile rainbow trout (*Oncorhynchus mykiss*) surviving a routine acute toxicity test (96 h LC₅₀) performed with graded concentrations of BKM and GM effluents. Secondly, we carried out a series of three laboratory exposures with adult Atlantic tomcod. In addition to measuring hepatic CYP1A1 mRNA from the tomcod, we also measured hepatic EROD activity as a second measure of CYP1A1 gene expression in one exposure. In another exposure plasma concentrations of the antioxidant vitamins E (tocopherol) and A (dihydroretinyl palmitate and retinyl palmitate) were also measured as an indicator of oxidative stress associated with CYP1A induction (Palace *et al.* 1998, 2004). Finally, we compared hepatic concentrations of CYP1A1 mRNA and plasma concentrations of

vitamins A and E in tomcod caged for 10 d directly in the plumes of the two pulp mills in the Miramichi estuary versus a site just upstream of the 1% effluent plume, and in fish held in clean water in the laboratory.

2.0 MATERIALS AND METHODS

2.1 RAINBOW TROUT COLLECTION AND MAINTENANCE

Sixty immature rainbow trout weighing $1.75\text{g} \pm 0.32$ wet weight (mean $\pm$ 1 Standard Error (S.E.)) and having an average total length of $6.00\text{ cm} \pm 0.46$ (mean $\pm$ S.E.) were obtained from a local hatchery in Fredericton N.B., Canada and were subjected to LC_{50} tests from June 7th to June 11th 1994 by Currie and Buchanan Environmental Consultants (Fredericton, N.B.). For the LC_{50} test, 5 fish were placed in individual 20 L plexiglass containers equipped with airstones, containing either BKME concentrations of 100, 50, 25, 12.5, 6.25 and 0% (v/v) or GME concentrations of 50, 25, 12.5, 6.25, 3.12 and 0% (v/v). Experiments began within 24 hours of collecting the effluents from the pulp and paper mills. Dilutions were made with spring water having a pH of 6.4 and water temperatures were maintained at 15°C. Solutions were not changed and the fish were not fed during the LC_{50} experiment. After a 133-hour effluent exposure, the 38 rainbow trout that survived the acute lethality LC_{50} test were frozen in liquid nitrogen for subsequent CYP1A1 mRNA analysis by I. Wirgin (New York University Medical Center; Tuxedo NY).

2.2 TOMCOD COLLECTION AND MAINTENANCE FOR LAB EXPERIMENTS

Atlantic tomcod were collected from commercial rainbow smelt (*Osmerus mordax*) bag nets set off Sheldrake Island in the lower estuary of the Miramichi River, New Brunswick, Canada (Fig. 2.2), on July 4 and October 12, 1994 (first and second experiments) and on March 2, 1995 (third experiment). The fish were transported to the Gulf Fisheries Centre in Moncton and maintained in 1200L aquaria with recirculating water. Salinities and temperatures for the three experiments respectively were: 2.0 - 6.0 ppt and 9.0 - 10.2 °C, 5.7 - 6.8 ppt and 8.5 - 9.0 °C, 4.8 - 7.0 ppt and 7.7 - 12.2 °C. The static system is depicted in Fig 2.1. All fish were inoculated with the antibiotic Baytril (enrofloxacin) to prevent outbreaks of atypical furunculosis, which is endemic in this population (Williams *et al.*, 1997a). Baytril has been shown to induce CYP1A1

gene transcription in tomcod (Williams *et al.* 1997b) but we assumed that its effects would be minimal following the 16-56 d acclimation period. The fish were fed once a day, either in the morning or early in the afternoon with 400 g chopped cod filets for 16 d before the first effluent experiment, 30 d before the second effluent experiment and 56 d before the third effluent experiment. The dates of the experiments were: July 20-26, 1994, November 11-17, 1994 and April 27 to May 5, 1995. The fish were not fed during the experiments.

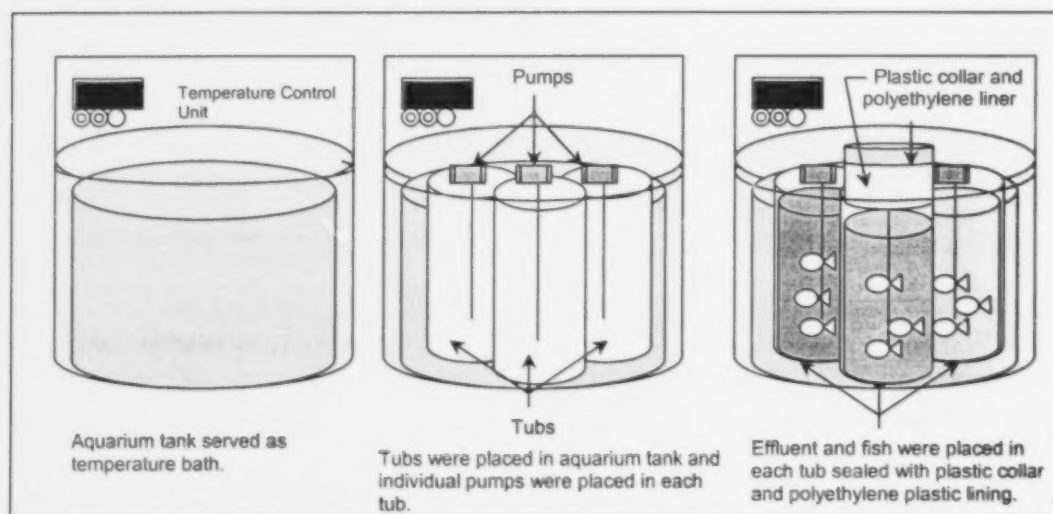


Figure 2.1: The static system used during the three effluent experiments. Tubs lined with polyethylene bags and topped with collars prevented fish from jumping were placed in aquarium tanks which served as temperature baths. Individual pumps re-circulated the water within each tub to maintain oxygen levels.

2.2.1 Effluent collection and setup

The groundwood mill effluent was collected from the groundwood mill reservoir located at Nelson Miramichi (see 2.3.1 for description of site and mill process). The initial water source at the beginning of the pulping process will be referred to as the groundwood raw water. The kraft mill effluent was collected from the secondary treatment facility at the kraft mill located at Newcastle. The initial water source at the beginning of the paper making process will be referred to as the kraft raw water. Both the kraft raw water and the groundwood raw water were tested to determine if they were sources of inducers. Prior to use, the effluents were stored in the laboratory in 140 L plastic tubs lined

with polyethylene bags. One hundred percent and 1% BKME concentrations were used in the first effluent experiment. More realistic BKME concentrations from 10% to 0.01% were used in the second and third effluent experiments as they better reflected the concentrations of the effluent in the receiving water (Martel *et al.* 1994). In the experiments with GME, a maximum concentration of 2% was used in the first study as concentrations greater than 3.12% were lethal to rainbow trout. The effluents were diluted with dechlorinated tap water, and 700 g of Instant Ocean™ salt was added to each tub for the first effluent and third experiment in order to maintain similar salinity with an average salinity of (5.4 ppt) and (5.5 ppt) for the first and third experiments respectively. For the second effluent experiment, all treatments were diluted with 10 ppt seawater obtained from Shediac, New Brunswick, Canada resulting in an average salinity of (6.2 ppt). The control group for the second effluent experiment was placed in seawater diluted with dechlorinated tap water to obtain 6.3 ppt salinity in a 180 L plastic tub lined with two polyethylene bags. Pumps were mounted at the water surface and drew water from the bottom of the tub. The objective in circulating water this way was to maintain high dissolved oxygen concentrations in the water without bubbling air which might alter solution chemistry through gas parging (W. Fairchild, Fisheries and Oceans, Moncton, New Brunswick, pers. comm.).

There were 12 tomcod per tub (treatment) in the first and second experiment (except for 20 tomcod in the case of the control) and 15 tomcod per tub in the third experiment. The third experiment had four control groups, one sampled at the start of the experiment (initial control; n=10), and three sampled at the end (tank 4, n= 15; tank 7, n=15; tank 2, n=12).

2.3 TOMCOD COLLECTION AND MAINTENANCE FOR CAGE EXPERIMENT

Atlantic tomcod were collected with smelt bag nets off Sheldrake Island in the lower estuary of the Miramichi River on September 22 and October 12, 1994 (Fig 2.2). All fish were transported to the Gulf Fisheries Centre in Moncton, N.B., Canada and maintained in 1200 L aquaria with recirculating water (Shediac Estuary seawater diluted to 10 ppt with distilled Moncton City water; 8.5 °C). All fish were inoculated with the

antimicrobial Baytril (enrofloxacin) 5-7 d after introduction to the aquarium (September 27 and October 19, 1994 respectively) to prevent expression of the atypical form of furunculosis, a disease endemic in this population (Williams *et al.*, 1997a). Fish were fed chopped cod filets *ad libitum* once daily.

2.3.1 Description of caging area

Two pulp and paper mills discharge effluent into the brackish, tidal waters of the upper Miramichi River estuary, one on the north shore just upstream of the part of Miramichi City formerly known as Newcastle, and the other on the south shore in the former town of Nelson (Fig. 2.2). Both mills are in the region of the confluence of the Northwest Miramichi and Southwest Miramichi tributaries.

The groundwood mill on the south shore began operation in 1964. At the time of this study (1994) the mill produced 272-336 air-dried metric tons (ADMT) per day of groundwood pulp which was used in production of lightweight coated paper at the Kraft Pulp and Paper Mill on the north shore. Bleaching used alkaline hydrogen peroxide and wood furnish was fir and spruce. Water was taken from Carding Mill Brook and effluent was discharged on-shore after primary treatment at a rate of 14,019 m³/d into the Southwest Miramichi River just downstream of the mill. The 1% effluent plume was found mainly in the upper 2 m of the water column and extended approximately 1 km by 250 m on a rising tide but was restricted to a much smaller area (ca. 100 m²) on a falling tide (JWEL 2000a; Fig 1). Effluent was acutely toxic to *Daphnia magna* (48 h LC50: 8.8-23%) and rainbow trout (*Oncorhynchus mykiss*) (96 h LC50: 2.2-16%).

The bleached kraft mill on the north shore began operation in 1948. At the time of this study it was producing ca. 1300 ADMT/d of kraft pulp and coated paper. The bleaching process consisted of chlorination, caustic extraction, and a hypochlorite and chlorine dioxide (CEHDED) process. Wood furnish was mainly softwood (balsam fir, spruce, pine) with a small amount of mixed hardwood (birch, maple, beech). Water was taken from the Southwest Miramichi River, upstream of tidal water, at Bryenton and was discharged after primary and secondary treatment (aerated lagoon) nearshore in the

Northwest Miramichi River at a rate of 73,000-75,000 m³/d. The 1% effluent plume was believed to extend ca. 2.2 km upriver on a rising tide and less than 100 m on a falling tide at the time of this study (JWEL 2000b). More recent studies indicate that the 1% plume extends ca. 1.4 km upstream and 1.7 km downstream and lower concentrations of this effluent are detectable considerably further both upstream and downstream (JWEL 2004a; Fig 1).

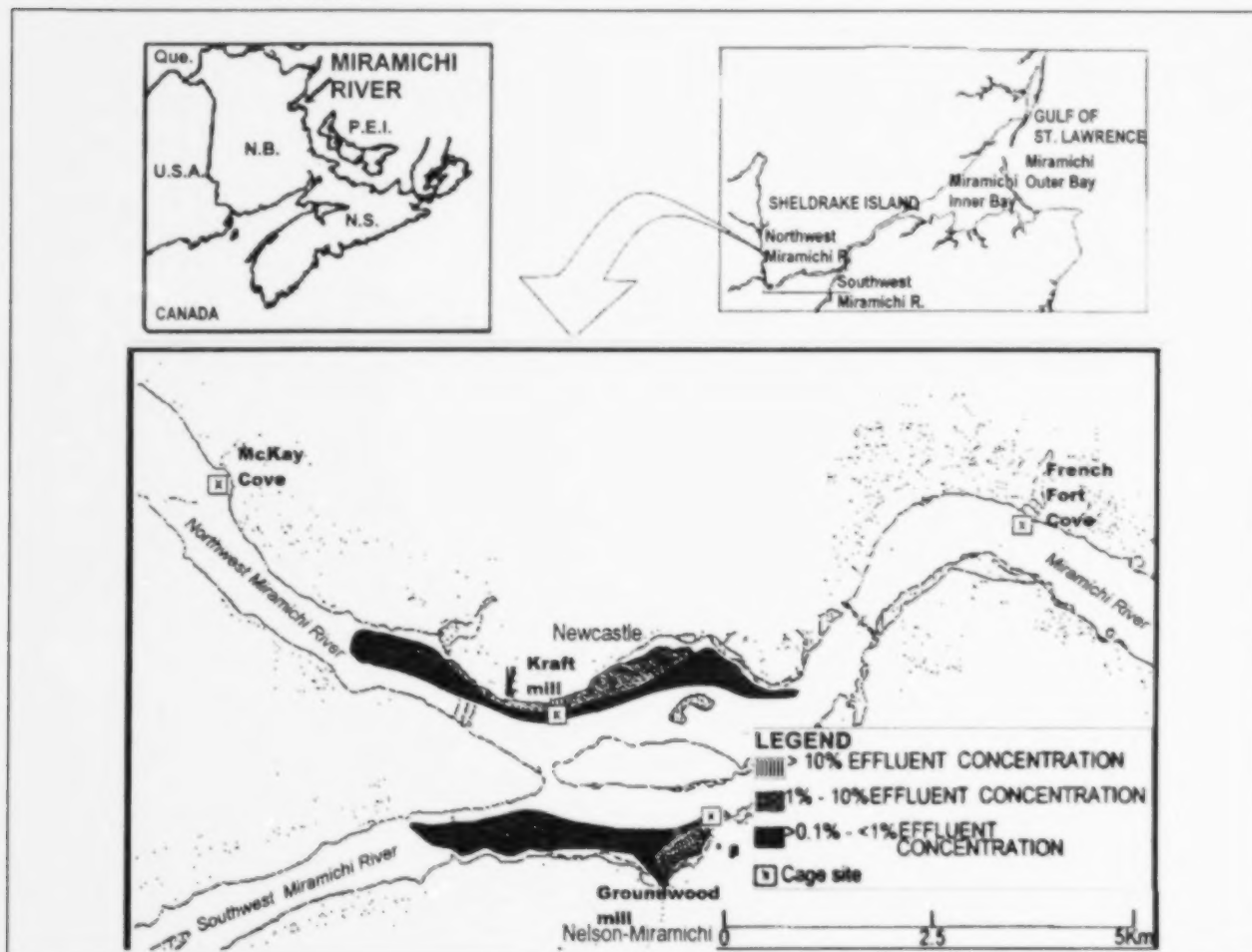


Figure 2.2: Map of Miramichi River indicating the four sites where Atlantic tomcod were caged (stars) and the collection site of fish using smelt bag nets off Sheldrake Island in the lower estuary of Miramichi River (modified from JWEL 2000a and 2004a).

Effluent, in 1994, was non-toxic to rainbow trout and *D. magna* except for samples collected in February, March and May which had LC50 values for *D. magna* of 97%, 92% and 84% respectively. Sublethal bioassays carried out with this effluent indicated

low inhibiting effect on early life growth of inland silverside (*Menidia beryllina*) but inhibition of reproduction in both sea urchin and marine alga (*Champia parvula*) at concentrations of 1-10% (JWEL 2000b).

2.3.2 Cage experiment

Tomcod were caged on October 25, 1994 at the four sites: kraft mill, groundwood mill, McKay Cove and French Fort Cove (Fig 2.2). Cages consisted of 1 m³ nylon mesh cages suspended within metal frames which were anchored on the bottom with weights and maintained upright with small floats (Fig 2.3).

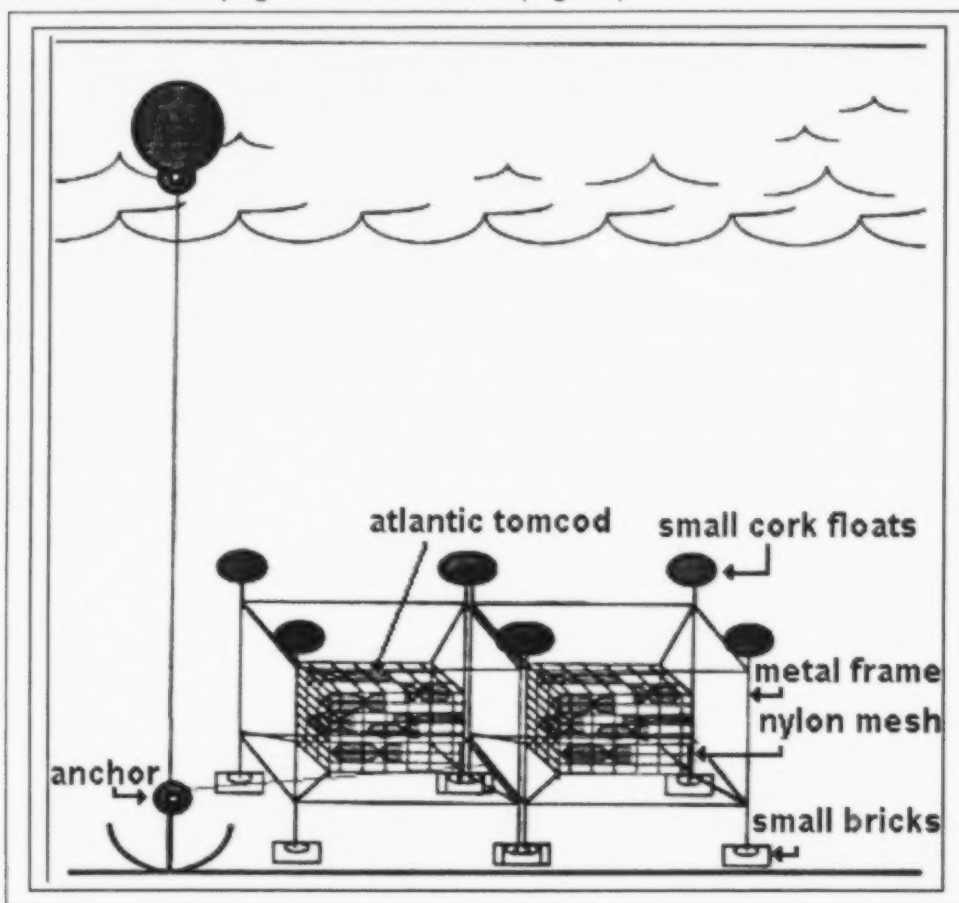


Figure 2.3: Layout of cages at study sites

Water depths, temperature and salinities at the beginning of the cage experiment were collected using a hand-held thermometer and refractometer from a bottom water sample

collected with a Van Dorn bottle (Table 2.1). Fifteen tomcod were placed in each cage and two cages were tied together at each site, for a total of thirty tomcod per site.

Table 2.1: Temperature, salinity and depth bottom readings taken during a falling tide at the cage sites Kraft Mill (KM), Groundwood Mill (GM), McKay Cove (MC) and French Fort Cove (FF) in the Miramichi River on October 25, 1994.

Site	Temperature (°C)	Salinity (ppt)	Depth (m)
KM	11	10	8
GM	11	10	6.5
MC	11	6	5
FF	10	10	4

Cages were not moved once they were anchored and fish were not fed from the time they were caged to the time they were sacrificed. Cages at the French Fort Cove site were not retrieved due to their illicit removal. Although 30 fish were caged at each site, sample sizes analyzed for CYP1A1 mRNA and vitamin A and E concentrations are less than 30 due to mortalities. In addition, CYP1A1 mRNA data were collected only from male tomcod because too few females were available to make a comparison at the end of the study (0-7 per site).

All tomcod were sacrificed with a blow to the head on November 4, 1994, 10 d after they had been caged in the Miramichi River. An additional control group of 30 tomcod held in clean aquarium water throughout the experiment was sacrificed on November 5, 1994. Fish livers were extracted and frozen in liquid nitrogen for subsequent analysis of CYP1A1 mRNA levels in the laboratory of Dr. Isaac Wirgin in Tuxedo, New York, U.S.A. Average lengths and weights of tomcod used in the cage experiment showed no significant difference between treatment groups in terms of lengths or weights of fish at the end of the experiment (ANOVA on $\log_{10}(X+1)$ transformed values, $p>0.05$). Mean (SE) lengths varied by treatment from 20.29 (0.61) cm – 21.36 (0.60) cm and mean weights varied from 65.17 (5.94) g – 70.93 (5.57) g.

2.4 CYP1A1 mRNA

2.4.1 Hepatic isolation

At the end of exposures, fish were killed with a blow to the head, weighed and measured. Average lengths and weights revealed no significant differences between treatment and control groups for any of the laboratory experiments with tomcod ($p > 0.05$, ANOVA). Mean lengths (SE) varied by treatment from 16.40 (0.60) cm – 21.00 (0.82) cm and mean weights varied from 28.45 (6.07) – 68.18 (9.56) g. Livers were extracted and frozen in liquid nitrogen with the exception of rainbow trout which were frozen whole and livers were subsequently extracted immediately before analysis. Liver samples were used for subsequent analysis of CYP1A1 mRNA levels and EROD activity in the laboratories of Dr. Isaac Wirgin (New York University Medical Centre; Tuxedo, NY), and Dr. Kelly Munkittrick (Environment Canada, National Water Research Institute, Burlington, Ontario) respectively.

2.4.2 RNA isolation

RNAzol reagent (Biotex Laboratories, Houston, TX) was used to isolate hepatic RNA from the fish sample. Approximately 20-80 mg frozen liver was homogenized using a teflon-coated mortar and pestle in 1.2 mL RNAzol reagent, a composite of 2-mercaptoethanol, phenol and guanidine thiocyanate as described in Chomczynski and Sacchi (1987). Chloroform (120 μ l) was added to the homogenate which was then put on ice for approximately 15 min, and then centrifuged at 4°C for 15 min at 16,000 x g. The supernatant was decanted in a 1.5 ml Eppendorf and an equal amount of isopropanol was added to it. After 3 h at -70 °C, the homogenate was then centrifuged at 4°C for 15 min at 16,000 x g. The supernatant was eliminated and the solid pellet and 100-130 μ l diethylpyrocarbonate-treated water was used to resuspend the samples. One-tenth volume of 5M NaCl was added along with ice-cold 100% EtOH. The sample was held at -70°C overnight before being centrifuged at 4°C for 30 min at 16,000 x g. The supernatant was discarded and the pellet resuspended in a volume of diethylpyrocarbonate-treated water which resulted in a concentration of 2-4 μ g/ μ l. RNA concentration. Purity of the

sample was determined by UV spectrophotometry using wavelengths between 260-280 nm in a Beckman DU-40 Spectrophotometer.

2.4.3 Northern blotting

Northern blot analyses were used to quantify hepatic CYP1A1 mRNA levels. A batch of denatured sample buffer was prepared by the addition of 2.4 ml formaldehyde, 7.5 ml deionized formamide, 1.5 ml 10 x (3-[N-morpholino] propanesulfonic acid) (MOPS), 1 ml diethylpyrocarbonate-treated water, 1 ml glycerol and 0.8 ml of 10% (w/v) bromophenol blue. The aliquots were stored at -20°C until needed. A total of 10 µg of each sample was denatured by adding 15 µl of sample buffer and incubating for 15 min at 65°C. Electrophoresis was carried out at 120 V for 2.0 - 2.5 h on 1.0% agarose gels (SeaKem GTG, FMC, Bioproducts) containing 1 x MOPS and 4% formaldehyde. After electrophoresis, a solution of 5 µg/ml ethidium bromide was used to stain the gels. In order to ensure equal loading and the integrity of the RNA, the rRNA bands were observed under UV light. The RNA was then transferred from Northern gels to Nytran nylon membranes (Schleicher & Schuell) through capillary action (Southern, 1975) using the following transfer medium, 10 x SSC (175.3 g NaCl, 88.2 g sodium citrate and water to 1 L, pH=7.0). After the transfer, the membranes were washed for 5 min in 2 x SSC and then baked for 2 h in a vacuum oven at 80°C.

2.4.4 Northern blot hybridization

Before hybridization, the membranes were washed for 1 h in 1x SSC/0.1% SDS (sodium dodecyl sulfate, pH =7.2) at 65°C. The Nytran membranes were then incubated in a prehybridized solution. This solution contains 2 ml of 50 x Denhardt's reagent (5 g polyvinylpyrrolidone, 5 g bovin serum albumin [fraction V, Sigma], (5 g of Ficoll [type 400, Pharmacia] and water to 500 ml), 5 ml of 20 x SSPE (175.3 g NaCl, 27.6 g NaH₂PO₄ • H₂O, 7.4 g EDTA and water to 1 L, pH=7.4), 12 ml double distilled water, 0.5 ml of 20% SDS and 0.4 ml of sheared and boiled salmon gonad DNA (10 mg/ml) for 2 h at 65°C. The prehybridized solution was then replaced with a hybridized solution. The hybridized solution contained 0.5 ml of 50 x Denhart's solution, 2.5 ml of 20 x SSPE, 6.55 ml double distilled water, 0.25 ml of 20% SDS, 1 g of dextran sulfate, 0.2 ml boiled salmon gonad

DNA and probe DNA. The CYP1A cDNA probe from a 3-MC induced rainbow trout (pP₁450-3' [Heilmann *et al.*, 1988]) was ³²P-radiolabelled using a nick translation kit (BRL-Gibco) as indicated in Rigby *et al.* (1977) and the CYP1A cDNA probe from a β NF induced tomcod was ³²P-radiolabelled using a nick translation kit (BRL-Gibco) as indicated in Rigby *et al.* (1977). Radiolabelling involved addition of 4 μ l of ³²P-dCTP and 5 μ l of a mixture of dATP, dTTP and dGTP to approximately 0.2 μ g of CYP1A DNA, (water and 5 μ l of DNA Polymerase I/DNase I) and the resulting mixture was brought up to a volume of 45 μ l. This mixture was incubated for 60 min at 15°C before the reaction was terminated with 45 μ l of 1 x TE (100 mM Tris, 10mM EDTA) buffer and 5 μ l stop buffer. The nick translated probe was passed through a Sephadex G-50 column. This column was made in a 1.5 ml Eppendorf tube capped with nylon wool in which the nick translated probe was spun for 2 min at 1500-2000 rpm.

After the membranes had been incubated in the hybridized solution at 65 °C overnight, they were washed twice at 20°C for 15 min in 6 x SSPE/0.1% SDS and once in 1 x SSPE/0.1% SDS (same temperature and duration). They were then washed twice at 65°C for 30 min in 6 x SSPE/0.1% SDS before being exposed at -80°C for 24-72h to Kodak XAR-5 or Fuji RX film with intensifying screens. The autoradiographs were then analyzed using a Millipore Biolume computer analysis system with the Whole Band Analysis package to determine the relative CYP1A1 mRNA levels.

2.4.5 Slot blotting

Each sample (10 μ l) was denatured by the addition of 0.6 x the RNA volume of 0.1 M NaH₂PO₄ (pH=6.5), 0.8 x the RNA volume of glyoxal and 2.5 x the RNA volume of dimethylsulfoxide (DMSO). Samples were then incubated for 1 h at 50°C before being brought up to a final salt concentration of 6-10 x SSC by the addition of 10 x SSC for a final volume of 200 μ l. Slot Blot apparatus (Schleicher & Schuell Minifold II) was assembled with a BA85 nitrocellulose membrane (Schleicher & Schuell) attached to a vacuum pump. Each slot that was to be loaded on the apparatus was rinsed three times with 200 μ l of 6 x SSC before and after the samples were loaded. Each slot was rinsed

three times with 200 μ l of 6 x SSC before the filter membrane was baked at 80°C for 2 h in a vacuum oven.

2.4.6 Slot blot hybridization

Nitrocellulose filters were incubated at 65°C for 3 h in a prehybridized solution of 0.15 g glycine, 0.75 ml of 1 M NaH_2PO_4 , 6 ml double distilled water, 3 ml of 50 x Denhardt's solution, 4.5 ml of 20 x SSC and 1 ml of sheared and boiled gonad DNA (10 mg/ml). As indicated by (Rigby *et al.*, 1977), the filters were hybridized to a radiolabelled CYP1A1 cDNA probe from a β NF-induced Atlantic tomcod. The hybridization occurred overnight in a solution consisting of 1.2 ml of 1M NaH_2PO_4 , 9 ml double distilled water, 4.5 ml of 20 x SSC, 0.6 ml of 50 x Denhardt's solution, 1.5 g dextran sulfate and 0.2 ml of sheared and boiled salmon sperm DNA (10 mg/ml) at 65°C. Membranes were washed three times in 1 x SSC/0.1% SDS at room temperature for 5 min and once in 0.1 x SSC/0.1% SDS at 65°C for 30 min. Membranes were then exposed to Kodak XAR-5 film with intensifying screens at -70°C. The CYP1A mRNA concentrations on the autoradiographs were determined by using the Whole Band Analysis package or a LKB Ultrascan XL laser densitometer on a Millipore Biolumage computer analysis system. Sample loading standardization was done in order to determine whether equal amounts of each sample were loaded on the northern and slot blots. Each membrane was stripped of the CYP1A1 probe with boiling double-distilled water and rehybridized to a rat (18S rRNA) probe, pHRR 118 (Chan *et al.*, 1984).

2.5 Vitamin isolation

Vitamin isolation was conducted for the second laboratory experiment and cage experiment using tomcod. Approximately 1 ml of blood per fish was extracted from its caudal vein, using 1cc preheparinized syringes. The blood was then transferred to a pre-labelled tube and centrifuged at 10,000 rpm for 10 min to obtain plasma. The supernatant plasma was then transferred to another pre-labelled Eppendorf tube and frozen in liquid nitrogen for subsequent vitamin A (didehydroretinyl palmitate and retinyl palmitate) and E (tocopherol) analysis by Dr. Scott Brown at the Freshwater Institute in Winnipeg, Manitoba, Canada.

2.5.1 Vitamin assay

Isocratic high-performance liquid chromatography (HPLC) was used to measure the tocopherol and retinoids (Palace and Brown, 1994). The tomcod plasma samples were kept on ice and in subdued light throughout the homogenization and extraction procedure. Blood plasma (~100 mg) was weighed and homogenized (Polytron) with the addition of 2 ml of distilled deionized water (Millipore®, Milli Q, Bedford, MA). HPLC grade ethanol (200 μ l) was added to the plasma homogenate (200 μ l) in order to precipitate proteins after homogenization. Tomcod plasma samples were extracted using 500 mL ethyl acetate/hexane; 3:20 (v/v) and the resulting residues were redissolved in 100 μ L mobile phase and 20 mL of residue solution was injected onto a 3 μ m bead size Adsorbosphere HS C₁₈ column. This column was 4.6 mm i.d. and 150 mm long, with an attached 10 mm Adsorbosphere guard column (Alltech Associates Inc., Deerfield, IL). The HPLC system consisted of a model 704 system controller, a four-channel model 620 data module (Gilson Medical Electronics, Milwaukee, WI), a model 231 automatic sample injector and two model 302 solvent pumps. A Gilson model 116 dual channel UV absorbance detector was set at 292 nm for tocopherol acetate and tocopherol detection, and at 325 nm for dehydroretinyl ester and dehydroretinol detection. As indicated by Rettenmaier and Schuep (1992), low tocopherol concentrations in samples were verified by using a Shimadzu model RF-535 fluorometric detector with fluorescence set at 295 nm excitation wavelength and 330 nm emission. Furthermore, a Shimadzu model RF-535 fluorometric detector was set at 480 nm emission wavelength; a 330 nm excitation wavelength was used for quantification of retinyl palmitate, retinol and retinyl acetate. The column was heated to and maintained at 26°C. Both samples and standards were eluted isocratically with acetonitrile/methanol/water; 70:20:10 (v/v/v) delivered at a flow rate of 1 ml/min. Retinyl acetate, retinyl palmitate, retinol, tocopherol and tocopherol acetate standards were purchased from Sigma Chemical Co. (St. Louis, MO).

By evaluating different absorbance/fluorescence properties, it is possible to infer that some unidentified chromatographic peaks are also vitamin A compounds (Spear et

al., 1992; Ndayibagira *et al.*, 1995). For example, the absorption spectrum of one of the peaks was scanned and the absorption spectrum was similar to that of didehydroretinol which colluded with didehydroretinyl palmitate standards (S. Brown, National Water Research Institute, Burlington, Ontario, pers. comm.). Precise identification of all these esters was not possible due to lack of exact standards, but the major dehydroretinyl ester (dRE2) concentration was approximated. The estimates were based on retinyl palmitate standards, including a correction factor for the different UV absorbances of dehydroretinol and retinol at 325 nm (Stancher and Zonata, 1984).

The internal standards used were retinyl acetate and tocopherol acetate. The recovery of these internal standards was used to correct for the efficiency of each extraction. Recovery efficiencies were determined by adding known amounts of tocopherol (875-4375 ng) and retinoids (8-400 ng). Tomcod blood plasma samples (~2.5-50 mg) were analyzed to examine the linearity between the amount of plasma extraction and the concentration of retinoids and tocopherol.

2.6 EROD analysis

EROD analysis was conducted on tomcod from the third laboratory experiment. Tomcod liver samples were thawed on ice and analyzed for Ethoxyresorufin-O-deethylase (EROD) activity. Samples were homogenized in 4 ml/g of HEPES-KCl (0.02 M HEPES, 0.15 M KCl, pH=7.5). The post-mitochondrial supernatant (PMS) was centrifuged at 12,000 x g for 20 min and was then transferred to 12 x 75 mm borosilicate glass tubes and frozen at -80 °C (Pohl and Fouts, 1980, as modified in Muir *et al.*, 1990). Samples were analyzed in triplicate with one blank. The reaction mixture contained 10 µl of magnesium sulfate (0.154 g/ml ddH₂O), 1250 µl of 0.1 M HEPES buffer (pH=7.8), 30 µl reduced nicotinamide adenine dinucleotide phosphate (NADPH; 55 mg/ml ddH₂O), 50 µl bovine serum albumin (BSA; 40 mg/ml ddH₂O), 50 µl of PMS and 20 µl of 7-ER (0.022 mg/ml DMSO) in 13 x 100 mm borosilicate glass tubes. Analyses were conducted at 25°C and the reaction was terminated after 2-4 min by adding 3 ml of methanol. Blanks consisted of 3 ml of methanol added to 20 µl of 7-ER. The tubes were centrifuged at 8000 to 8400 x g for 5 min. The resulting supernatant

was transferred to cuvettes and analyzed using a spectrofluorometer (Perkin Elmer LS50), with an emission wavelength of 585 nm and an excitation wavelength of 530 nm. The emission slit width was set at 20 nm and the excitation slit width was set at 2.5 nm. The results were converted to pmol resorufin mg protein/min using a standard curve.

2.7 Statistical analysis

D'Agostino's test was used to check for normality (Zar, 1984). Log transformations ($\log_{10}[X+1]$ transformed data) were successful in correcting for non-normal distributions. Homogeneity of variances was evaluated using F-max test (Sokal and Rohlf, 1981). Log transformation was also necessary when heterogeneity of variance was found.

When statistical assumptions were met, a two-factor ANOVA was used to analyze CYP1A1 mRNA levels, vitamin concentrations (dihydroretinyl palmitate, retinyl palmitate, tocopherol) and (EROD) enzyme activity (Simstat 3.5c, Montreal, QC). If only one sex was analyzed, a one factor ANOVA was used (Statistix 3.5, 1991). Where ANOVA assumptions were violated a two- or one-factor Kruskal-Wallis test was used instead. When both sexes gave identical results, a one-way ANOVA or Kruskal-Wallis test was used. Multiple comparisons were conducted using a Tukey test. Bivariate data were analyzed using a student T-test when variances were homogenous or could be successfully transformed (F-test Sokal and Rohlf, 1981) Otherwise a nonparametric Mann-Whitney test was used (Zar, 1984). Correlation between CYP1A1 mRNA levels and EROD activity (third experiment) were investigated using a Spearman rank correlation test.

3.0 RESULTS

3.1 HEPATIC CYP1A1 MRNA CONCENTRATIONS IN RAINBOW TROUT SURVIVING LC50 TEST

3.3.1 CYP1A1 mRNA

Two control groups were pooled for comparison with other treatments since no significant difference in their hepatic CYP1A1 mRNA concentrations was detected ($T_{(2),7} =$

0.117; two-sample t-test $p=0.8825$). Juvenile trout exposed for 133 h to BKME at concentrations of 12.5%, 25%, 50% and 100% (v/v) all showed significantly higher CYP1A1 mRNA concentrations than controls ($H=15.92$; $p=0.0014$; $p<0.05$ non-parametric multiple comparison; Table 3.1). There was no significant CYP1A1 mRNA induction in rainbow trout exposed to 3.12% GME effluent ($U_{(5,9)}=30$; Mann-Whitney test $p=0.1918$; Table 3.1) and mortality of all trout exposed to higher concentrations of GME precluded assessing their CYP1A1 induction.

Table 3.1: Relative mean concentrations of hepatic CYP1A1 mRNA levels in juvenile rainbow trout (*Oncorhynchus mykiss*) surviving the LC_{50} test using 100, 50, 25, 12.5, 6.25 and 0% (v/v) bleached kraft mill effluent (BKME), 50%, 25%, 12.5%, 6.25%, 3.12 and 0% (v/v) groundwood mill effluent (GME) from the two pulp mills operating on Miramichi River, N.B., Canada.

Treatment	n	n/a	m ^o	MRNA ^a	Fold induction over control	Significant
BKME 100%	5	0	0	24.19 ± 1.320	10.34	*
BKME 50%	5	0	0	23.82 ± 6.111	10.18	*
BKME 25%	4	0	1	23.49 ± 4.171	10.04	*
BKME 12.5%	5	0	0	13.01 ± 5.169	5.56	*
BKME 6.25%	4	0	1	4.02 ± 3.599	1.72	
GME 50%	0	0	5	ϕ	ϕ	
GME 25%	0	0	5	ϕ	ϕ	
GME 12.5%	0	0	5	ϕ	ϕ	
GME 6.25%	0	0	5	ϕ	ϕ	
GME 3.12%	5	0	0	9.26 ± 3.635	3.96	
Control ^b	9	1	0	2.34 ± 0.673	—	

* Significant difference than control $p<0.05$

n = Sample size after LC_{50} test

n/a = Not analyzed

m^o = Number of mortalities

^a Mean ± standard error; values are in optical density (O.D.) units.

^b Control = GME 0% & KME 0%

ϕ = No data due to 100% mortality

3.2 FIRST LABORATORY EXPERIMENT WITH TOMCOD

3.2.1 CYP1A1 mRNA:

Levels of CYP1A1 mRNA were significantly induced, 6-fold relative to laboratory controls, in tomcod held for 6 d in 100% bleached kraft mill effluent (BKME) but not in tomcod held in either 1% BKME or kraft raw water ($F_{(3,32)}=32.99$; one-factorial ANOVA; sexes combined, $p=0.0001$, data $\log_{10} [X+1]$ transformed, Table 3.2). Data from males

and females were pooled for this analysis as no significant difference was detected between them. CYP1A1 mRNA levels in male and female tomcod exposed to groundwood mill effluent (GME) were analyzed separately as levels were significantly higher in females than males ($F_{(1,17)}=8.785$; two-factorial ANOVA, $p=0.008$, data $\log_{10} [X+1]$ transformed). Much lower concentrations of GME than BKME were tested in this experiment (2% vs 100%) because of GME's much higher acute toxicity. Furthermore, CYP1A1 mRNA levels were not quantified from tomcod exposed to 2% GME due to misplaced samples, but there was no significant CYP1A1 mRNA induction in either male ($F_{(2,11)}=0.46$; one-factorial ANOVA, $p=0.6452$, data $\log_{10} [X+1]$ transformed) or female ($F_{(2,6)}=0.22$; one-factorial ANOVA, $p=0.8110$, data $\log_{10} [X+1]$ transformed) tomcod exposed to 0.2% GME or groundwood raw water (Table 3.2).

Table 3.2: Relative mean hepatic CYP1A1 mRNA levels in male, female, and combined male and female Atlantic tomcod (*Microgadus tomcod*) during the first exposure (July 20-26, 1994) to 1% and 100% (v/v) bleached kraft mill effluent, 2% and 0.2% (v/v) groundwood mill effluent, 100% (v/v) kraft raw water and 100% (v/v) groundwood raw water from Miramichi, N.B., Canada.

Treatment group	N				mRNA ^a			
	M	F	N/A	M ^b	Male	Female	Combined	Fold induction over control
Kraft 100%	4	7	1	0	7.54 ± 1.555	5.19 ± 0.702	6.05 ± 0.761*	6.58*
Kraft 1%	7	2	3	0	1.18 ± 0.267	1.67 ± 0.722	1.28 ± 0.247	1.39
Kraft raw water	5	2	5	0	1.01 ± 0.326	0.78 ± 0.482	0.95 ± 0.252	1.03
Groundwood 2%	—	—	—	—	—	—	—	—
Groundwood 0.2%	4	4	3	1	0.63 ± 0.286	1.27 ± 0.318	0.95 ± 0.232	-
Groundwood raw water	4	2	6	0	0.92 ± 0.273	1.25 ± 0.295	1.03 ± 0.201	-
Lab. Control	6	3	2	1	0.62 ± 0.192	1.51 ± 0.243	0.92 ± 0.206	

* Significant difference from control $p < 0.05$

^a Mean ± standard error; values are in optical density (O.D.) units.

— No CYP1A1 mRNA data

M = male tomcod that survived the exposure and were analyzed for CYP1A1 mRNA

F = female tomcod that survived the exposure and were analyzed for CYP1A1 mRNA

N/A = tomcod that survived the exposure but were not analyzed

M^b = number of tomcod that did not survive the exposure

3.3 SECOND LABORATORY EXPERIMENT WITH TOMCOD

3.3.1 CYP1A1 mRNA:

Male tomcod exposed for 6 d to 3% GME showed significantly higher levels of hepatic CYP1A1 mRNA than laboratory controls ($F_{(3,30)}=4.14$, one-factorial ANOVA, $p=0.0144$ data $\log_{10} [X+1]$ transformed; Table 3.3). Female tomcod were not analyzed for CYP1A1 mRNA levels because there were too few of them. Mortalities were high in the tomcod exposed to the two highest concentrations of GME (5/12 for each of 3% and 0.3%) and were even higher among the tomcod exposed to 10% BKME (10/12) (Table 3.3). Significant CYP1A1 mRNA induction was seen in the two surviving male tomcod exposed to 10% BKME ($F_{(4,33)}=6.89$; one-factorial ANOVA, $p=0.0004$, data $\log_{10} [X+1]$ transformed), Table 3.3). Lower concentrations of GME and BKME produced lower mortality rates and no significant CYP1A1 mRNA induction.

Table 3.3: Relative mean hepatic CYP1A1 mRNA levels in male Atlantic tomcod (*Microgadus tomcod*) during the second effluent (November 11-17, 1994) exposure to 0.01%, 0.1%, 1% and 10% (v/v) kraft and 0.03%, 0.3% and 3% (v/v) groundwood mill effluent from Miramichi, N.B., Canada.

Treatment group	n			mRNA ^a	Fold induction over control	Significant
	M	N/A	m ^o			
Kraft 10%	2	0	10	18.30 ± 8.264	5.53	*
Kraft 1%	7	2	3	4.93 ± 1.924	1.49	
Kraft 0.1%	6	3	3	2.02 ± 0.868	-	
Kraft 0.01%	9	1	2	3.15 ± 1.167	-	
Groundwood 3%	6	1	5	20.01 ± 7.013	6.04	*
Groundwood 0.3%	6	1	5	8.89 ± 1.891	2.69	
Groundwood 0.03%	8	3	1	7.53 ± 2.000	2.28	
Lab. Control	14	6	0	3.31 ± 0.836	-	

* Significant difference from control $p < 0.05$

^a Mean ± standard error; values are in optical density (O.D.) units.

M = male tomcod that survived the exposure and were analyzed for CYP1A1 mRNA

N/A = tomcod that survived the exposure but were not analyzed

m^o = number of tomcod that did not survive the exposure

3.3.2 Vitamin levels:

Blood plasma concentrations of didehydroretinyl palmitate were significantly lower in tomcod held for 6 d in 3% and 0.3% GME than in controls ($H=16.74$ Kruskal-Wallis sexes combined; $p=0.0008$; data $\log_{10} [X+1]$ transformed). While didehydroretinyl palmitate levels were similarly depressed in the two tomcod surviving exposure to 10% BKME, the small sample size precluded detection of a statistical difference (Table 3.4). Retinyl palmitate levels were not significantly affected by exposure to either BKME or GME in either males or females ($p>0.05$, Kruskal-Wallis, data $\log_{10} [X+1]$ transformed; Table 3.5). However, retinyl palmitate levels were significantly lower in male tomcod exposed to 0.3% GME than in those exposed to 0.03% GME ($H=9.404$; Kruskal-Wallis, $p=0.0117$; data $\log_{10} [X+1]$ transformed). Tocopherol levels were significantly higher in males than females ($F_{(1,36)}=6.919$; two-factorial ANOVA, $p=0.012$; data $\log_{10} [X+1]$ transformed) but were not significantly affected by exposure to BKME or GME in either sex (Table 3.6).

3.4 THIRD LABORATORY EXPERIMENT WITH TOMCOD

3.4.1 CYP1A1 mRNA:

Since there were relatively more female than male tomcod, hepatic CYP1A1 mRNA levels in the third effluent experiment were analysed only for female tomcod. Two of four groups of control fish were analysed to determine CYP1A1 mRNA levels. These two groups of control fish were sampled from tanks 4 and 7 and were treated most similarly to the other treatment groups by being held in tubs for the entire duration of the experiment and sacrificed at the end of the experiment. Data for the two control groups were pooled for further analysis as they did not differ significantly ($T\text{-test}_{(2),10}=1.339$, two-sample t-test, $p>0.05$; Table 3.7).

Table 3.4: Relative mean concentration of vitamin A (dihydroretinyl palmitate) in blood plasma of male, female and combined male and female Atlantic tomcod (*Microgadus tomcod*) during the second effluent experiment (November 11-17, 1994) exposed to 0.01%, 0.1%, 1% and 10% (v/v) kraft and 0.03%, 0.3%, 3% (v/v) groundwood mill effluent from Miramichi, N.B., Canada.

Treatment group	N				dihydroretinyl palmitate ^a		
	M	F	N/A	M ^b	Male	Female	Combined
Kraft mill effluent 10%	2	0	0	10	9.53 ± 0.535 [†]	---	9.53 ± 0.535
Kraft mill effluent 1%	6	1	2	3	34.24 ± 6.193	19.64 ± 0.000	32.16 ± 5.634
Kraft mill effluent 0.1%	6	3	0	3	23.58 ± 5.294	15.13 ± 1.685	20.76 ± 3.728
Kraft mill effluent 0.01%	9	0	1	2	48.90 ± 8.783	---	48.90 ± 8.783
Groundwood mill effluent 3%	6	1	0	5	12.84 ± 4.336	10.80 ± 0.000	12.55 ± 3.676 *
Groundwood mill effluent 0.3%	7	0	0	5	11.85 ± 1.763	---	11.85 ± 1.763 *
Groundwood mill effluent 0.03%	8	3	0	1	36.33 ± 6.610	26.44 ± 5.295	33.63 ± 5.071
Lab control	13	6	1	0	37.23 ± 5.060	15.07 ± 2.884	30.23 ± 4.278

* Significant difference from control $p < 0.05$

^a values are Mean vitamin concentration in $\mu\text{g/ml} \pm$ standard error.

[†] Although vitamin concentration appears to be significantly lower than in laboratory controls, multiple comparison tests did not show significance, owing to the small sample size.

M = male tomcod that survived the exposure and were analyzed for vitamin assay

F = female tomcod that survived the exposure and were analyzed for vitamin assay

N/A = tomcod that survived the exposure but were not analyzed

m^b = number of tomcod that did not survive the exposure

Table 3.5: Relative mean concentration of vitamin A (retinyl palmitate) in blood plasma of male, female and combined male and female Atlantic tomcod (*Microgadus tomcod*) during the second effluent experiment (November 11-17, 1994) exposed to 0.01%, 0.1%, 1% and 10% (v/v) kraft and 0.03%, 0.3%, 3% (v/v) groundwood mill effluent from Miramichi, N.B., Canada.

Treatment group	n				retinyl palmitate ^a		
	M	F	N/A	m ^o	Male	Female	Combined
Kraft mill effluent 10%	2	0	0	10	0.65 ± 0.130	---	0.65 ± 0.130
Kraft mill effluent 1%	6	1	2	3	12.67 ± 3.888	4.60 ± 0.000	11.51 ± 3.482
Kraft mill effluent 0.1%	6	3	0	3	7.31 ± 3.815	1.76 ± 0.436	5.46 ± 2.634
Kraft mill effluent 0.01%	9	0	1	2	17.92 ± 4.690	---	17.92 ± 4.690
Groundwood mill effluent 3%	6	1	0	5	5.34 ± 1.436	2.09 ± 0.000	4.87 ± 1.299
Groundwood mill effluent 0.3%	7	0	0	5	2.97 ± 0.680	---	2.97 ± 0.680
Groundwood mill effluent 0.03%	8	3	0	1	12.00 ± 2.229	5.02 ± 2.547	10.10 ± 1.962
Lab control	13	6	1	0	9.92 ± 1.652	2.16 ± 0.885	7.47 ± 1.652

* Significant difference from control $p < 0.05$

^a values are Mean vitamin concentration in µg/ml ± standard error.

M = male tomcod that survived the exposure and were analyzed for vitamin assay

F = female tomcod that survived the exposure and were analyzed for vitamin assay

N/A = tomcod that survived the exposure but were not analyzed

m^o = number of tomcod that did not survive the exposure

Table 3.6: Relative mean concentration of vitamin E (tocopherol) in blood plasma of male, females and combined male and female Atlantic tomcod (*Microgadus tomcod*) during the second effluent experiment (November 11-17, 1994) exposed to 0.01%, 0.1%, 1% and 10% (v/v) kraft and 0.03%, 0.3%, 3% (v/v) groundwood mill effluent from Miramichi, N.B., Canada.

Treatment group	n				Tocopherol ^a		
	M	F	N/A	m ^o	Male	Female	Combined
Kraft mill effluent 10%	2	0	0	10	5.72 ± 4.470	---	5.72 ± 4.470
Kraft mill effluent 1%	6	1	2	3	113.49 ± 45.933	1.25 ± 0.000	97.46 ± 42.001
Kraft mill effluent 0.1%	6	3	0	3	84.36 ± 70.220	9.12 ± 4.334	59.28 ± 47.046
Kraft mill effluent 0.01%	9	0	1	2	127.35 ± 47.427	---	127.35 ± 47.427
Groundwood mill effluent 3%	6	1	0	5	63.51 ± 31.613	29.26 ± 0.000	58.62 ± 27.162
Groundwood mill effluent 0.3%	7	0	0	5	17.31 ± 11.999	---	17.31 ± 11.999
Groundwood mill effluent 0.03%	8	3	0	1	221.05 ± 147.991	16.61 ± 8.408	165.29 ± 109.465
Lab control	13	6	1	0	112.04 ± 45.121	6.83 ± 2.023	78.82 ± 32.587

* Significant difference from control $p < 0.05$

^a values are Mean vitamin concentration in µg/ml ± standard error.

M = male tomcod that survived the exposure and were analyzed for vitamin assay

F = female tomcod that survived the exposure and were analyzed for vitamin assay

N/A = tomcod that survived the exposure but were not analyzed

m^o = number of tomcod that did not survive the exposure

Table 3.7: Relative mean hepatic CYP1A1 mRNA levels in female Atlantic tomcod (*Microgadus tomcod*) used as controls in the third effluent exposure (April 27 to May 5, 1995) using a static system. Control groups (tank 4 and tank 7) included tomcod, which were not exposed to pulp mill effluent and were sacrificed at the end of the experiment.

Control group	N			CYP1A mRNA ^a	Significant
	F	N/A	m ^o		
Tank 4	4	8	3	0.48 ± 0.081	
Tank 7	8	7	0	0.73 ± 0.125	
Tank 2	--	12	0	--	--
Initial control	--	10	0	--	--
Control #16 ^b	12	37	3	0.65 ± 0.092	

$\alpha = 0.05$

^a Mean ± standard error; values are in optical density (O.D.) units.

^b Control #16 includes (tank 4 and 7)

F = female tomcod that survived the exposure and were analyzed for CYP1A1 mRNA

N/A = tomcod that survived the exposure but were not analyzed

m^o = number of tomcod that did not survive the exposure

No significant induction of CYP1A1 mRNA over controls was observed in female tomcod exposed for 8 d to BKME of up to 10%, GME of up to 3%, or the raw waters used by either pulp mill (Table 3.8). The only significant difference observed in this data set was, in fact, a depression of CYP1A1 mRNA levels in female tomcod exposed to 1% BKME relative to kraft raw water ($H=3.14$; Kruskal-Wallis, $p=0.0149$; data $\log_{10} [X+1]$ transformed; Table 3.8).

Table 3.8: Relative mean hepatic CYP1A1 mRNA levels in female Atlantic tomcod (*Microgadus tomcod*) during the third effluent exposure (April 27 to May 5, 1995) to 100% kraft raw water, 0.01%, 0.1%, 1% and 10% (v/v) kraft and 100% groundwood raw water, 0.03%, 0.3% and 3% (v/v) groundwood mill effluent from Miramichi, N.B., Canada.

Treatment group	n			mRNA ^a	Fold activity over control	Significant
	F	N/A	m ^o			
Kraft 10%	8	6	1	1.73 ± 0.804	2.66	
Kraft 1%	9	5	1	0.43 ± 0.044	-	
Kraft 0.1%	11	2	2	0.55 ± 0.079	-	
Kraft 0.01%	8	7	0	0.52 ± 0.122	-	
Kraft raw water	9	6	0	1.23 ± 0.221	1.89	
Groundwood 3%	4	10	1	0.73 ± 0.159	1.12	
Groundwood 0.3%	6	9	0	0.44 ± 0.094	-	
Groundwood 0.03%	9	6	0	0.50 ± 0.119	-	
Groundwood raw H ₂ O	7	8	0	0.71 ± 0.124	1.09	
Control #16 ^b	12	15	3	0.65 ± 0.092	-	

* Significant difference from control $p < 0.05$

^a Mean ± standard error; values are in optical density (O.D.) units.

^b Control #16 includes (tank 4 and 7)

F = female tomcod that survived the exposure and were analyzed for CYP1A1 mRNA

N/A = tomcod that survived the exposure but were not analyzed

m^o = number of tomcod that did not survive the exposure

3.4.2 EROD activity:

EROD levels were similar between male and female tomcod not exposed to pulp mill effluents (i.e., controls), but levels differed among the four control groups ($F_{(3,42)}=5.19$, one-factorial ANOVA sexes combined, $p=0.039$). EROD levels appeared to drop over the course of the experiment, being significantly lower in two of the three groups sampled at the end of the experiment than in the initial control ($p<0.05$, Tukey test, Table 3.9).

Table 3.9: Relative mean levels of EROD activity in male, female and combined male and female Atlantic tomcod (*Microgadus tomcod*) used as controls in the third effluent exposure (April 27 to May 5, 1995) using a static system. Control groups included tomcod which were not exposed to pulp mill effluent and were sacrificed at the beginning of the experiment (initial control); the other control groups (tank 4, 7 and 2) were not exposed to pulp mill effluent and were sacrificed at the end of the experiment.

Control group	n				EROD activity ^a			
	M	F	N/A	m ^o	Male	Female	Combined	Fold induction
Tank 4	7	4	1	3	0.86 ± 0.101	0.99 ± 0.262	0.91 ± 0.108*	1.26*
Tank 7	7	6	2	0	0.31 ± 0.144	0.60 ± 0.215	0.44 ± 0.128	-
Tank 2	6	6	0	0	0.61 ± 0.130	0.60 ± 0.169	0.60 ± 0.101	-
Initial control	3	7	0	0	0.87 ± 0.322	1.10 ± 0.142	1.03 ± 0.132*	1.39*
Control #15 ^b	23	23	3	3	0.63 ± 0.086	0.82 ± 0.101	0.72 ± 0.067	

* Significant difference between controls (tank 7 & tank 2 ≠ tank 4 & initial control, $p < 0.05$)

^a Mean ± standard error; values are in pmol/mg protein/min.

^b Control #15 includes (tank 2,4,7 and the initial control)

M = male tomcod that survived the exposure and were analyzed for EROD activity

F = female tomcod that survived the exposure and were analyzed for EROD activity

N/A = tomcod that survived the exposure but were not analyzed

m^o = number of tomcod that did not survive the exposure

Nevertheless, because changes were small and not universal, a combined group of all controls was used for comparison with the other treatment groups sacrificed at the end of the experiment. Compared against this pooled control, EROD activity was not significantly induced in either sex by exposure to $\leq 3\%$ GME, $\leq 10\%$ BKME, or the water used by the groundwood mill ($p > 0.05$, ANOVA, $\log_{10}(X+1)$ transformed data; Table 3.10). However, EROD activity was significantly higher in tomcod exposed to the intake water used by the Kraft mill than in controls ($F_{(5,108)} = 5.58$, one-factorial ANOVA sexes combined, $p = 0.0002$; data $\log_{10}[X+1]$ transformed;).

Table 3.10: Relative mean levels of EROD activity in male, female and combined male and female Atlantic tomcod (*Microgadus tomcod*) during the third effluent exposure (April 27 to May 5, 1995) to 100% kraft raw water, 0.01%, 0.1%, 1% and 10% (v/v) kraft and 100% groundwater raw water, 0.03%, 0.3% and 3% (v/v) groundwater mill effluent from Miramichi, N.B., Canada.

Treatment Group	N				EROD activity ^a			
	M	F	N/A	m ^c	Male	Female	Combined	Fold induction over control
Kraft 10%	4	10	0	1	1.40 ± 0.323	1.02 ± 0.323	1.13 ± 0.348	1.57
Kraft 1%	5	9	0	1	0.48 ± 0.241	0.98 ± 0.170	0.80 ± 0.149	1.11
Kraft 0.1%	2	11	0	2	0.52 ± 0.377	0.71 ± 0.123	0.68 ± 0.113	-
Kraft 0.01%	7	8	0	0	0.79 ± 0.323	0.26 ± 0.089	0.51 ± 0.167	-
Kraft raw water	6	9	0	0	1.24 ± 0.437	2.24 ± 0.438	1.88 ± 0.338*	2.61*
Groundwood 3%	10	4	0	1	0.91 ± 0.105	1.03 ± 0.174	0.94 ± 0.088	1.31
Groundwood 0.3%	5	10	0	0	0.69 ± 0.154	0.86 ± 0.120	0.81 ± 0.094	1.12
Groundwood 0.03%	6	9	0	0	0.65 ± 0.172	0.77 ± 0.181	0.72 ± 0.126	1.00
Groundwood raw H ₂ O	7	8	0	0	0.96 ± 0.459	0.54 ± 0.092	0.73 ± 0.215	1.01
Control #15 ^b	23	23	3	3	0.63 ± 0.086	0.82 ± 0.101	0.72 ± 0.067	

* Significant difference from control $p < 0.05$

^a Mean ± standard error; values are in pmol/mg protein/min.

^b Control #15 includes (tank 2,4,7 and the initial control)

M = male tomcod that survived the exposure and were analyzed for EROD activity

F = female tomcod that survived the exposure and were analyzed for EROD activity

N/A = tomcod that survived the exposure but were not analyzed

m^c = number of tomcod that did not survive the exposure

Among the fish sampled for both CYP1A1 mRNA and EROD there was no significant correlation between the two measures of CYP1A1 activity (Spearman Rank Correlation, $r_{s0.05(2)(82)}=0.217$; $r_s=0.175$, data $\log_{10} [X+1]$ transformed; $p>0.05$).

3.5 CAGE EXPERIMENT WITH TOMCOD

3.5.1 CYP1A1 mRNA

Some tomcod from all cage sites died during the 10 d caging, but more died at the kraft mill site (20/30) than at the groundwood mill (4) or the upstream reference site at McKay Cove (5). Cages at the downriver reference site (French Fort Cove) were not recovered so no data were gathered from these fish. Male tomcod caged at the kraft mill site (KM) groundwood mill site (GM) and the McKay Cove (MC) cage site showed significantly higher levels of hepatic CYP1A1 mRNA than laboratory controls (Kruskal-Wallis, $H=20.368$, $p=0.0001$, Table 3.11), but non-parametric multiple comparison tests did not detect any difference among the three cage sites.

Table 3.11: Relative median concentrations of hepatic CYP1A1 mRNA (integrated optical density units) in male Atlantic tomcod (*Microgadus tomcod*) caged for 10 in the Miramichi River, N.B., Canada.

Treatment group	n		mRNA	Fold induction over control	Significant
	Male	n/a			
Kraft mill	10	0	12.30	5.3	*
Groundwood mill	15	11	17.25	7.4	*
McKay Cove	15	10	10.35	4.4	*
French Fort Cove	φ	φ	φ	φ	φ
Laboratory control	15	15	2.33		

* Significantly greater than control $p < 0.05$, Kruskal Wallis test.

n/a = number of samples not analyzed

φ = data not analyzed due to lost cage

3.5.2 Vitamin levels

Blood plasma concentrations of didehydroretinyl palmitate were significantly lower in tomcod caged for 10 d at GM and MC than in laboratory controls ($F_{(3,85)}=7.95$; one-factorial ANOVA, sexes combined; $p=0.0001$; $\log_{10} (X+1)$ transformed data; Table 3.12). Retinyl palmitate levels were significantly lower in tomcod caged at GM than at KM ($F_{(3,85)}=3.52$; one-factorial ANOVA, sexes combined; $p=0.0183$; $\log_{10} (X+1)$ transformed data; Table 3.13) but neither site had significantly different results from the control group. Tocopherol levels were significantly higher in males than in females ($F_{(1,81)}=10.108$; two-factorial ANOVA; $p=0.003$; $\log_{10} (X+1)$ transformed data; Table 3.14). but were not significantly different among treatments for either males ($F_{(3,60)}=1.00$; one-factorial ANOVA; $p=0.3996$; $\log_{10} (X+1)$ transformed data) or females ($F_{(2,21)}=0.79$; one-factorial ANOVA; $p=0.4681$; $\log_{10} (X+1)$ transformed).

Table 3.12: Relative mean ($\pm$ standard error) concentration of vitamin A (didehydroretinyl palmitate; $\mu\text{g/ml}$) in male (M), female (F) and combined male and female Atlantic tomcod caged for 10 d in the Miramichi River, N.B., Canada.

Treatment group	n		didehydroretinyl palmitate		
	M	F	Male	Female	Combined
Kraft mill	9	0	17.50 ± 2.253	—	17.50 ± 2.253
Groundwood mill	19	7	10.76 ± 2.571	7.94 ± 1.844	$10.00 \pm 1.939^*$
McKay Cove	19	5	9.36 ± 1.825	8.52 ± 2.619	$9.18 \pm 1.522^*$
French Fort Cove	ϕ	ϕ	ϕ	ϕ	ϕ
Laboratory control	17	13	24.73 ± 4.225	17.70 ± 2.242	21.68 ± 2.627

* Significantly less than control, $p < 0.05$ ANOVA

ϕ = data not analyzed due to lost cage

Table 3.13: Relative mean ($\pm$ standard error) concentration of vitamin A (retinyl palmitate; $\mu\text{g/ml}$) in male (M), female (F) and combined male and female Atlantic tomcod caged for 10 d in the Miramichi River, N.B., Canada.

Treatment group	n		retinyl palmitate		
	M	F	Male	Female	Combined
Kraft mill	9	0	7.02 ± 1.357	---	7.02 ± 1.357
Groundwood mill	19	7	3.34 ± 0.974	1.47 ± 0.432	2.84 ± 0.734
McKay Cove	19	5	3.41 ± 1.037	2.39 ± 1.539	3.20 ± 0.871
French Fort Cove	ϕ	ϕ	ϕ	ϕ	ϕ
Laboratory control	17	13	7.41 ± 2.063	5.14 ± 1.918	6.04 ± 1.426

* Significantly less than control $p < 0.05$, ANOVA

ϕ = data not analyzed due to lost cage

Table 3.14: : Relative mean ($\pm$ standard error) concentration of vitamin E (tocopherol; $\mu\text{g/ml}$) in male (M), female (F) and combined male and female Atlantic tomcod caged for 10 d in the Miramichi River, N.B., Canada.

Treatment group	n		tocopherol		
	M	F	Male	Female	Combined
Kraft mill	9	0	146.84 ± 43.067	---	146.84 ± 43.067
Groundwood mill	19	7	121.91 ± 31.827	29.48 ± 13.787	97.02 ± 24.749
McKay Cove	19	5	74.43 ± 16.065	9.078 ± 4.588	60.82 ± 13.831
French Fort Cove	ϕ	ϕ	ϕ	ϕ	ϕ
Laboratory control	17	13	130.41 ± 35.058	40.72 ± 17.183	93.30 ± 23.008

* Significantly different than control $p < 0.05$, ANOVA

ϕ = data not analyzed due to lost cage

4.0 DISCUSSION

The hypothesis tested in this study was that the effluents discharged by two pulp and paper mills into the upper Miramichi River estuary were responsible for the elevated

hepatic CYP1A1 mRNA concentrations reported in Miramichi fish by previous studies (Courtenay et al. 1993, 1994, 1995; Wirgin et al. 1994).

The preliminary experiment with rainbow trout provided equivocal support for this hypothesis. Rainbow trout exposed to Miramichi's BKME showed dose-responsive CYP1A1 mRNA induction at effluent concentrations of 12.5% and higher. These results do not necessarily imply that lower concentrations of BKME will not induce trout but rather, given the small sample size of 4 fish per treatment, there was insufficient statistical power to detect small differences. It may be that longer exposure than the 133 h used in this experiment might produce induction at lower concentrations of effluent than we observed. However, the results from this experiment suggest that response to concentrations of BKME of 1% or less would be very small. Since very little of the Miramichi estuary contains over 1% effluent (see Fig 2.2), the implication of these results is that few fish in the estuary would show CYP1A1 response to the pulp mill and only fish living near the BKME discharge pipe would show elevated CYP1A1 activity. The bleached kraft mill discharges effluent into shallow water onshore and produces a plume of $\geq 1\%$ effluent onshore that is 70-400 m wide and extends upstream up to 1400 m on flood tides and downstream up to 1700 m on ebb tides (JWEL 2004a). Some reports of elevated CYP1A1 mRNA or EROD activity have come from fish sampled within this area (Courtenay et al. 1993; Courtenay and Couillard 1998) but others have come from fish sampled downriver, far beyond the 1% effluent plume (Wirgin et al. 1994).

The only groundwood mill effluent concentration that yielded survivors that could be analyzed for CYP1A1 induction was 3.12% which produced a 4-fold induction over controls. This was not statistically significant but again the sample size was small. Nevertheless, current results suggest that further experiments with larger sample sizes should be conducted at lower concentrations. The mortality associated with 6.25% and higher concentrations of groundwood mill effluent reflects acute lethality of that effluent at the time of this experiment (1994). Toxicity of pulp mill effluents has decreased in Canada over the past decade and at this particular mill only occasional mortalities in rainbow trout

LC₅₀ tests are now observed and those are generally associated with high ammonia concentrations (JWEL 2004b).

The results of this study confirm the presence of CYP1A1 inducers for fish in Miramichi BKME as previous studies have postulated (Courtenay *et al.*, 1993, 1995; Wirgin *et al.*, 1994). However, the fact that significant induction was seen only in response to relatively high concentrations of BKME ($\geq 12\%$) suggests either that the species used in this experiment (rainbow trout) is much less sensitive to the CYP1A1 inducers in BKME than the species described in the earlier publications (Atlantic tomcod), or that other sources of CYP1A1 inducers must exist in the Miramichi River estuary. These two possibilities were explored through laboratory bioassays with Atlantic tomcod exposed to graded doses of pulp mill effluents, and through cage studies in the field.

Laboratory bioassays suggested that Atlantic tomcod are not much more sensitive to the CYP1A1 inducers in Miramichi pulp mill effluents than rainbow trout. Pure BKME produced significant CYP1A1 mRNA induction in male and female tomcod during the first exposure as did 10% BMKE in males of the second exposure. In each case the induction was comparable in magnitude (6-fold) to that observed in rainbow trout (6-10 fold). Neither species responded significantly to concentrations of BKME lower than 10%. Interestingly, exposure to 3% groundwood mill effluent also produced a significant 6-fold CYP1A1 mRNA induction in male tomcod of the second experiment. The 6-fold CYP1A1 inductions observed in this study are modest compared to hundreds-fold inductions reported in laboratory exposures with i.p. injections of PAH's (Courtenay *et al.* 1999) but are comparable to the 4-fold induction observed in caged tomcod in 1990 near the kraft mill and at Newcastle (Courtenay *et al.* 1993). Similarly, tomcod caged near the kraft mill in 1992 showed 11-fold induction over tomcod caged upriver (Courtenay *et al.* 1993) and wild tomcod sampled from Miramichi River in 1992 showed 14-fold CYP1A1 mRNA induction over tomcod from Margaree River and 8-fold over St. Lawrence and Saco / Royal River tomcod (Wirgin *et al.* 1994).

In a repeat of the exposure to $\leq 10\%$ BKME and $\leq 3\%$ GME, a third experiment with tomcod failed to produce significant induction in CYP1A1 mRNA or to increase EROD activity. Differences between experiments two and three included the sex of the fish examined (male vs female respectively) and reproductive state (pre-spawning vs post-spawning respectively) – both of which have been shown to affect CYP1A1 mRNA expression (Courtenay *et al.* 1994; Williams *et al.* 1998). However, it is also possible that the different biological responses reflect differences in effluent quality. The bleached kraft mill at Miramichi dredged its lagoons in the spring of 1994, which could have re-suspended some old sediments producing a more toxic BKME for some period of time (R. Parker, Environmental Protection Branch, Fredericton, New Brunswick, pers. comm.). This more toxic effluent produced mortality in several of the monthly LC50 tests performed with *Daphnia* in 1994 and might have contributed to the CYP1A1 induction observed in tomcod in the first two exposures which used effluent collected in July and November. By the following spring though, when the third experiment was conducted, effluent of the bleached kraft mill was no longer acutely toxic (Parker and Smith 1999). A third possibility is that decreased responsiveness in experiment three may have resulted from the longer pre-treatment acclimation period given these fish (56 d vs 16 d and 30 d in experiments one and two respectively). Other scientists have reported that fish adapted to polluted environments sometimes grow and survive less well under "optimal" laboratory conditions than conspecifics from cleaner environments (e.g., Meyer and Di Giulio 2003). Therefore laboratory conditions may have proven stressful to Miramichi tomcod.

Although the primary objective of this series of experiments was to determine whether or not Miramichi pulp mill effluents contained CYP1A1 mRNA inducers, we also measured vitamin stores as a supplementary measure of oxidative stress in the second experiment. Both treatments which elicited significant hepatic CYP1A1 mRNA induction in males, 10% BKME and 3% GME, also depressed didehydroretinyl palmitate (DP) concentrations in blood plasma, though a small sample size precluded statistical significance of this depression for 10% BKME. Lower concentrations of each effluent which did not induce CYP1A1 mRNA also did not depress DP titres with the exception

of 0.3% GME. We suspect that larger sample sizes would reveal an even closer association between these two responses to xenobiotic exposure. In contrast, we observed no indication of depressed titres of the other form of vitamin A (retinyl palmitate) or vitamin E (tocopherol). This discrepancy may be a result of the instability of these molecules relative to didehydroretinyl palmitate which renders them more susceptible to degradation through laboratory manipulations and physiological stress (S. Brown, National Water Research Institute, Burlington, Ontario, pers. comm.).

Decreases in blood plasma levels of retinoids have been reported in fish exposed to CYP1A1-inducing contaminants (Delorme *et al.*, 1994; Palace and Brown, 1994; Ndayibagira *et al.*, 1995). Lower retinoid concentrations have also been observed in fish exposed to pulp mill effluent (Friesen *et al.*, 1994) and in Atlantic tomcod collected from the Miramichi River (Fairchild *et al.* 1994). Vitamins are antioxidants and fish depend on tightly regulated supplies of vitamins to combat oxidative stressors. In general, when fish are exposed to oxidative stressors, an accelerated metabolism and breakdown of both retinoids and metabolites results in a greater demand for vitamin A (Gilbert *et al.*, 1995). Normal vitamin A homeostasis depends on an adequate supply of vitamin to target tissues and the enabling of cells to produce the functionally active forms of the vitamins (Zile, 1992). Vitamin E is also considered an important cellular compound against oxidative damage (Serbinova *et al.*, 1991; Roberfroid and Calderon, 1995).

We conclude from the laboratory assays that both BKME and GME from Miramichi's pulp mills contain CYP1A1 inducers for fish at least some of the time. The differences in CYP1A1 responses of tomcod between experiments suggest that responsiveness of tomcod to these inducers may be impaired at times by other chemicals in the effluents, by endogenous physiological factors not controlled in these experiments, or more likely that the concentrations of inducers in effluents may be temporally variable. In addition, the relatively small levels of gene induction produced by high concentrations of effluent lead us to suspect that these effluents are not solely responsible for the induction previously reported in wild tomcod of the Miramichi River, a conclusion supported by results of the caging experiment.

Tomcod caged for 10 d within the 1% plumes of both pulp mills showed significant CYP1A1 mRNA induction over fish retained in clean water in the laboratory. The 5-fold induction observed in the present study is comparable to the 3- to 4-fold induction reported in tomcod caged for 9 d at the BKME site and just downriver at Newcastle in 1990 by Courtenay *et al.* (1993). These results are consistent also with responses to high concentrations of the pulp mill effluents observed in the laboratory. However, tomcod caged beyond the 1% effluent plume upstream in McKay Cove also showed significant CYP1A1 induction. It is conceivable that some pulp mill effluent reaches McKay Cove (NATECH Environmental Services Inc. 2002), or did in previous years and left residues in the substrate, but it seems unlikely that concentrations would be high enough to explain the CYP1A1 response observed in the tomcod caged there. This is likely true even if the caged tomcod were particularly sensitive to CYP1A1 inducers as they may have been because of their treatment with the antibiotic enrofloxacin (Baytril). Williams *et al.* (1997b) reported that tomcod co-injected with Baytril and PCB congener 77 (3,3',4,4' tetrachlorobiphenyl) showed higher CYP1A1 mRNA induction than from PCB treatment alone and these effects persisted for at least 14 d. Fish in our experiment were caged only 6 d after Baytril injection, so might have experienced modulation of CYP1A1 expression during much or all of the caging period. However, while these other factors may have played some small role, we suspect that the most likely explanation for the induction observed in McKay Cove tomcod is that there are other sources of CYP1A1 inducers in the Miramichi River estuary.

Taken together, we conclude from the results of the laboratory assays and cage experiment in the river that the pulp and paper mill effluents contained CYP1A1 inducers for fish, at least some of the time, but probably at concentrations too low to explain the induction previously reported in fish throughout the Miramichi estuary. This observation may be helpful in interpreting results of a 4 year biomonitoring program carried out between September 1993 and September 1996 (Williams *et al.* 1998). During that program, hepatic CYP1A1 mRNA concentrations and a number of other morphometric and physiological parameters, were measured each spring and fall from tomcod in five estuaries of the southern Gulf of St. Lawrence. The objective was to test the utility of

these biomarkers for measuring exposure and early physiological response of fish to organic contaminants. The Miramichi was one of three industrialized estuaries sampled and tomcod were taken from the Loggieville area approximately 26 km downriver from the pulp and paper mills. The results of the present study would suggest that the pulp mill effluents would have a relatively minor impact on CYP1A1 mRNA levels seen in fish at this point in the estuary.

Finally, it should be noted that the results of the present study reflect conditions a decade ago (1994-1995). Since then, there have been a number of changes to the pulp and paper mills on the Miramichi River. For example, the effluent of the groundwood mill now undergoes secondary treatment before being discharged from a multiport bottom diffuser in the mid-channel of the Southwest Miramichi R. near the downstream end of Beaubear's Island. Consequently its 1% plume is much smaller than it was in 1994 (Fig 2.2) and the effluent is no longer acutely toxic (UPM-Kymmene Miramichi 2002; JWEL 2004b). Similarly, a number of process changes have been implemented at the bleached kraft mill. Total suspended solids, biological oxygen demand and volume of effluent have all decreased in recent years (JWEL 2004a) and a pilot plant which used ethanol for delignification (Alcell®) at the time of this study was shut down in 1996. In addition, potential influences of other anthropogenic effluents have changed. For example, a municipal wastewater treatment facility began discharging treated sewage just downstream from the BKM (Strawberry Marsh) in December 1997. Therefore the relative influence on Miramichi's two pulp and paper mills on CYP1A1 induction in fish may be different now than it was a decade ago. Nevertheless, the present operators of the mills, UPM-Miramichi, are currently interested in identifying practical sublethal toxicity tests for effluent monitoring (Riebel and Maclean 2002) and perhaps CYP1A1 mRNA induction or didehydroretinyl palmitate might serve as valuable biomarkers.

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